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The purpose of this work was to develop a novel system to study recruitment of factors, especially those involved in chromatin modification and remodeling, to an estrogen-responsive promoter. Transgenic fruitflies were created that conditionally express estrogen receptors and that contain a reporter whose expression is dependent on the estrogen receptor. Six doubly transgenic lines were developed. These transgenic flies express the estrogen receptor in response to heat shock and the reporter (green fluorescent protein) in response to estrogens. For five of six lines, estrogen-dependent expression of the reporter in salivary glands has been confirmed. Whole mounts of glands from control and estrogen-fed larvae were done and estrogen receptor visualized by immunofluorescence. Results suggest that much of the estrogen receptor in both control and hormone-treated larvae, while nuclear, is not bound to DNA. Chromosomal squashes of polytene chromosomes reveal that the chromatin-bound estrogen receptor is bound to euchromatic DNA and excluded from heterochromatic DNA. Surprisingly, estrogen made no difference in the apparent binding of receptor to DNA. These results suggest that estrogen receptors are bound to active regions of chromatin even in the absence of hormone. This may facilitate the search for high affinity binding sites when hormone is present.

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Table of Contents

Cover....1

SF 298....2

Table of Contents....3

Introduction....4

Technical Objectives....4

Key Research Accomplishments....6

Reportable Outcomes....7

Conclusions....7

References....

Appendices....8

INTRODUCTION

This research project was supported by a Concept Award and represents the work stemming from one year of effort. The aim of the research project was to develop a novel system for the elucidation of molecular details of the action of estrogen receptor with a particular focus on delineation of the timing and nature of the factors that are recruited to an estrogen regulated promoter. To achieve this it was proposed to create lines of transgenic fruitflies with a reporter gene regulated by estrogen response elements and that expressed estrogen receptor. The concept was to take advantage of the endoduplication that results in the polytene chromosomes characteristic of specific tissues such as the salivary gland of the developing fly. Once these double transgenic lines were developed, each had to be verified for the ability to respond to estrogens, particularly in the salivary gland. In the responsive lines, the binding of factors to specific chromosomal sites can be directly visualized by straightforward immunohistochemical techniques on chromosomal squashes from salivary glands. The ultimate goal of this research is to delineate a detailed kinetic and compositional analysis of the events that occur at an estrogen-responsive gene following the binding of the hormone-bound estrogen receptor to the promoter of a target gene.

BODY

As a Concept Award the proposal had no formal Statement of Work. The research accomplishments that addressed to goals outlined in the proposal will be addressed. At the time the proposal was written we had developed lines of transgenic fruitflies that contained sequences encoding the estrogen receptor (ER) under the control of the heat shock 70 promoter and had mapped the chromosomal locations of the transgenes. We had also developed lines of flies that contained a reporter gene (green fluorescent protein, GFP) under the control of a promoter regulated by estrogen response elements (EREs) and had mapped their chromosomal locations. Our initial efforts funded by this award were to demonstrate that the ER-transgenic flies could indeed express ER following heat shock and to optimize the heat shock and recovery conditions. An example of estrogen receptor expression in salivary glands from heat shocked larvae is shown in Figure 1A. Receptor staining is confined to the nucleus. The parental line of flies from which the transgenic flies are derived (yellow white, YW) exhibit no staining under the same conditions (Figure 1B). Two lines of flies, 1.3 and 3.2, that expressed ER robustly in response to a 90 min incubation of the larvae at 37C (heat shock conditions for fruitflies) were selected. These lines contain the ER gene on different chromosomes (II and III, respectively). These lines were bred to appropriate balancer lines as were the lines containing the reporter genes. Line 1.3 was then crossed to lines 11.1 and 11.2 containing the reporter gene on a different chromosome than the ER gene. This was done to prevent any possible interference between the loci when it came time to do the staining of chromosome squashes. ER line 3.2 was crossed to four reporter lines where the reporter is again on a different chromosome from the ER. In total then we have 6 doubly transgenic lines with a heat shock regulated ER gene and a ERE regulated GFP reporter gene. The properties of these are shown in the table included with the Reportable Outcomes section.

Once the doubly transgenic lines were developed, we assessed whether the lines were, in fact, responsive to estrogens. Larvae for each of the 6 lines were grown on medium with our without estrogen. Larvae from estrogen-fed and control vials were heat shocked for 90 min at 37C. Another set of estrogen-fed larvae was not heat shocked. Larvae were then allowed to recover for various times and GFP expression was assessed by fluorescence microscopy. Heat shocked larvae grown on estrogen medium exhibit GFP expression whereas heat shocked flies grown on control medium exhibited only the autofluorescence typical of fruitfly larvae. Flies that were not heat shocked to increase ER expression but were grown on estrogen-containing medium occasionally exhibit a small amount of GFP fluorescence. This is likely due to a small basal level of ER expression from the heat shock 70 promoter. Figure two shows fluorescence of three larvae. The top one is heat shocked, grown on control medium. The middle one is heat shocked, grown on estrogen medium, the bottom larva was not heat shocked but grown on estrogen medium. Only the middle larva exhibits substantial GFP expression establishing that GFP expression is dependent on hormone and expression of ER. We have determined that all 6 double transgenic lines express GFP in a receptor- and hormone-dependent manner.

Not all tissues expressed GFP equally well in response to estrogens. Also, although all lines express GFP in response to estrogen, not all do in the same pattern or to the same degree. Therefore, we felt it necessary to assess hormone-dependent GFP expression in salivary glands. Thus far we have confirmed by dissecting glands from larvae grown on control and estrogen medium that two lines exhibit robust GFP expression in salivary glands in response to hormone. See table in Reportable Outcomes. Three lines exhibit moderate or patchy estrogen-induced GFP expression. One line does not appear to express GFP in salivary glands even though there is substantial expression elsewhere in the larvae.

In another set of experiments salivary glands have been dissected from 3rd instar larvae that had been heat shocked and allowed to recover and express ER for 4 hours. The glands were placed in medium containing an estrogen for 30 minutes then whole mounts prepared for immunostaining. High resolution fluorescence microscopy reveals that estrogen receptor is nuclear and that much of it is in the extrachromosomal domains of the nucleus. Exposure to an estrogen doesn't really change this distribution. The only areas of the nucleus where no receptor staining is seen are heterochromatic regions.

The P.I. spent one spend a month in the laboratory of John Lis at Cornell University in order to learn chromosome squash techniques and to do some initial high resolution staining to look at recruitment of factors to the estrogen-dependent gene locus in July, 2002. The results obtained during this time were somewhat surprising. Estrogen receptor staining was readily apparent on chromosomes from estrogen-treated glands (fig. 3A). However, it was not simply associated with the single target gene locus or a few loci but rather receptor was present throughout the euchromatic DNA. Indeed, staining for ER was virtually coincident with that for RNA polymerase II (fig. 3B and overlap fig. 3C). The relative intensity of ER and RNA polymerase II differed but virtually every band that stained with one also stained with the other to some degree. Staining with DAPI (figure 3D) visualizes the regions of heterochromatic DNA. Overlapping the colors reveals that DAPI and ER staining are mutually exclusive (data not shown).

The most surprising finding of these data is shown in figures 3E and 3F. Even in the absence of estrogen treatment, ER was found associated with euchromatin. Overall, staining was indistinguishable from that seen in hormone-treated glands. Figure 3E shows staining for ER in chromosome squashes from untreated salivary glands and figure 3F shows the overlap in staining with RNA polymerase II. These data suggest that substantial amounts of ER is associated with DNA even in the absence of hormone and we speculate that this might be important mechanistically for the receptor to quickly find its specific target sites in the genome. Further work is needed to assess whether the limited sites of high intensity receptor staining represent ER target sites, particularly the ERE in the transgene, and whether the staining at the ERE is indeed increased by hormone. Nonetheless, we are positioned to write for further funding to pursue the use of this system to answer fundamental questions about ER action.

KEY RESEARCH ACCOMPLISHMENTS

- Developed immunohistochemical and Western blot methodologies for detection of estrogen receptor (ER) in fruitfly larvae and dissected salivary glands.
- Screened hsp70:ER transgenic flies for those lines that have highest expression of ER.
- Optimized heat shock conditions to achieve high ER expression.
- Selected two lines, 1.3 and 3.2, for crossing to transgenic fly strains containing reporter genes under the control of estrogen response elements.
- Crossed ER transgenic lines with 6 independent, mapped, reporter lines.
- Assayed all six double transgenic lines to confirm that the reporter gene, green fluorescent protein (GFP) is expressed in larvae in response to heat shock (to induce estrogen receptor) and an estrogenic hormone.
- Confirmed hormone-dependent expression of GFP in salivary glands dissected from larvae derived from five of the six strains.
- Performed immunohistochemical detection and fluorescence microscopy on whole mounts of salivary glands. Determined that much of ER, while nuclear, is not associated with chromatin. Hormone-treatment of salivary glands in vitro or hormone fed to intact larvae did not cause an apparent distribution of the ER. The only area of the nucleus from which ER was specifically excluded appeared to be regions of heterochromatic DNA.
- Spent one month in the laboratory of John Lis at Cornell University to learn chromosome squash technology in order to assess the recruitment of factors to the estrogen-responsive reporter genes.
- Performed chromosome squashes and immunohistochemical staining for estrogen receptor and RNA polymerase II. Estrogen receptor present throughout euchromatic DNA in the presence <u>and absence</u> of hormone.

REPORTABLE OUTCOMES

This work was presented at the ERA of Hope meeting in September 2002. A copy of the abstract has been appended.

The following 6 strains of transgenic fruitflies that are responsive to estrogens have been developed:

Strain name	Chromosomal Location of ER Transgene	Chromosomal Location of GFP Reporter Gene	Confirm express GFP Larvae	Confirm express GFP Salivary gland
1.3/11.1	II	III	+	+
1.3/11.2	II	III	+	++
3.2/10.5	III	II	+	++
3.2/10.6	III	II	+	+
3.2/4.2	III	X	+	-
3.2/8.1	III	X	+	+

CONCLUSIONS

We have completed the development of an estrogen-responsive reporter system and shown that we can detect a specific association of ER with active chromatin. Some technical hurdles remain before the system can be adequately exploited to examine the estrogen receptor-dependent recruitment of chromatin remodeling and modification factors as well as components of the basal transcription complex. The precise chromosomal locations of the transgenes need to be mapped in conjunction with ER immunohistochemistry on chromosome squashes to see if quantitatively increased ER binding at the ERE in the presence of hormone can be visualized. Other approaches that could be useful in conjunction with these studies are chromatin immunoprecipitation assays to independently confirm ER loading at the ERE and microarray assays to identify endogenous genes that may be regulated by ER. We have some data which implies that ER does modulate expression of some endogenous genes. With these studies, we are poised to be able to ask detailed questions about the kinetics of the recruitment of these different factors. This is a potentially powerful system that could be used to address questions on the molecular mechanisms of estrogen receptor action that are much more difficult or impossible to do in mammalian systems.

APPENDIX 1

Abstract presented at the Era of Hope meeting

Curing Breast Cancer in Fruitflies. What are you nuts?!

Steven K. Nordeen, Richard Heyer, Joan Hooper University of Colorado Health Sciences Center, Denver, CO 80262

Estrogens play a critical role in the development and growth of the mammary gland. Estrogen antagonist therapy is widely used in the treatment of estrogen-dependent tumors and as a prophylactic in women at high risk for breast cancer. Rational intervention with estrogen signaling is handicapped by the limited understanding of the molecular mechanisms involved. In order to understand the regulatory mechanisms of the estrogen receptor, it is critical to elucidate the factors that play a role in ER-dependent transcription. The specific goal of this Concept Award is to elucidate the nature and timing of the factors recruited to estrogen responsive promoters in response to hormone. To do so, a novel approach has been developed that exploits properties of the chromosomes of the fruitfly, Drosophila melanogaster. The polytene chromosomes of the salivary gland permit direct immunocytochemical detection and localization of factors associating with distinct sites on chromatin. We have engineered several lines of flies that conditionally express ER under the control of the heat shock promoter and additional lines of transgenic flies with ERE-containing transgenes at different chromosomal sites. These lines have been crossed to produce doubly transgenic flies with both the ER and the ER-responsive transgenes. Currently we are assessing hormone-regulation of the transgenes in the different strains. Once expression is confirmed we will perform in situ hybridization on chromosomal spreads to visualize the location of the transgenes in different lines. The amplification and loose packing of non-centromeric chromatin in the polytene chromosomes will allow direct visualization of the recruitment of factors to the transgenes including the receptor itself. We will then assess the recruitment of a variety of factors that are involved or suspected to be involved in steroid receptor-mediated transcription including RNA polymerase II, TATA binding protein, chromatin remodeling factors such as Brahma, Acf1, and ISW1, histone acetyltransferases, and other putative coactivators by immunohistochemical detection using antibodies to the Drosophila versions of these factors. In addition we will assess the state of histone modification at the promoter. The results of these studies will be presented. These studies will help to provide the fundamental information necessary if we are to understand the subtleties of estrogen receptor action necessary to fully exploit hormone-based therapies for breast cancer.

Personnel receiving pay from the research effort

Xia Guo, Professional Research Assistant

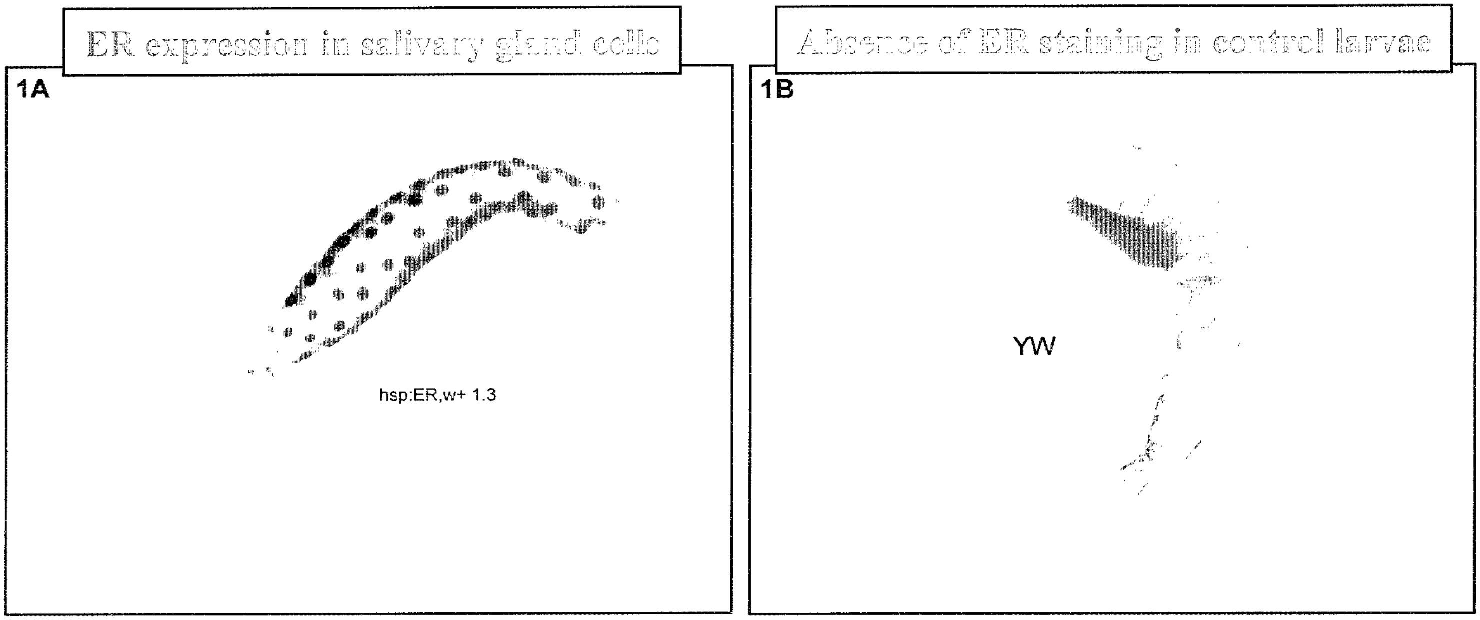
APPENDIX 2

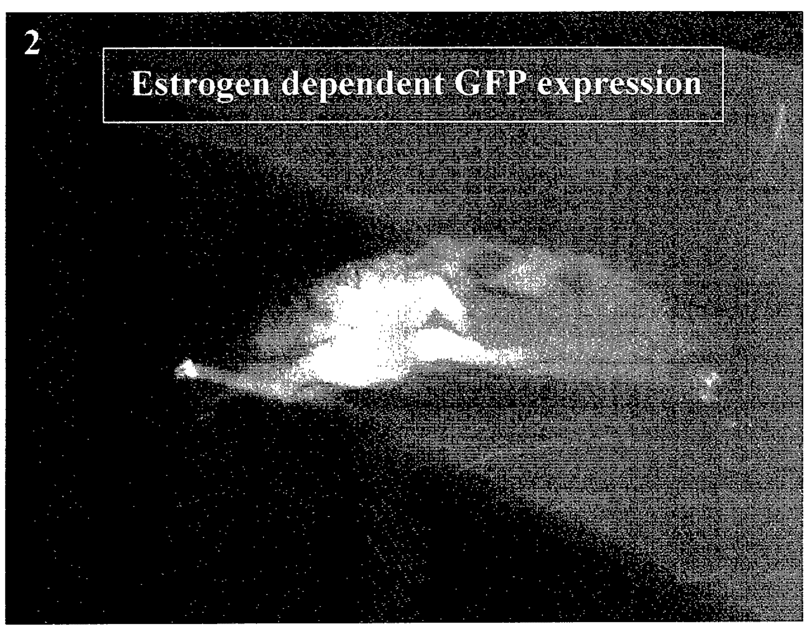
Figure Legends

Figure 1. Expression and cellular localization of estrogen receptor in the salivary gland of transgenic fruitflies. A. Larvae of transgenic fly strain 1.3 were subjected to a 90minute heat shock at 37C then allowed to recover for 4 hours at room temperature to express the estrogen receptor. Salivary glands were dissected, fixed, and stained for human estrogen receptor. B. Same as A except non-transgenic flies were used.

Figure 2. Expression of GFP in a hormone- and receptor-dependent fashion in 3rd instar larvae of doubly transgenic flies. Three larvae are shown in the field of the dissecting fluorescence microscope. The top larva was heat shocked to induce estrogen receptor expression and grown on control medium. It exhibits only dim autofluorescence. The middle larva was heat shocked and grown on medium containing an estrogen. The flies were grown overnight following heat shock. The bottom larva was also grown on medium containing estrogen but was not heat shocked. Estrogen receptor expression in this line is quite low.

Figure 3. Colocalization of ER and RNA polymerase II on the polytene chromosomes of salivary glands both in the presence and absence of estrogen. A. Immunofluorescence of ER on polytene chromosomes prepared from estrogen-treated glands. B. Co-staining for RNA polymerase II on the same chromosome squash. C. Overlap of the staining shown in A and B. D. DAPI staining for heterochromatic DNA on the same chromosome squash from estrogen-treated glands. E. ER immunofluorescence from a control gland not treated with estrogen. F. ER + RNA polymerase II immunofluorescence on polytene chromosomes from a control salivary gland not treated with estrogen.


ER expression in salivary gland cells
1A
hsp:ER,w+ 1.3
Absence of ER staining in control larvae
1B
YW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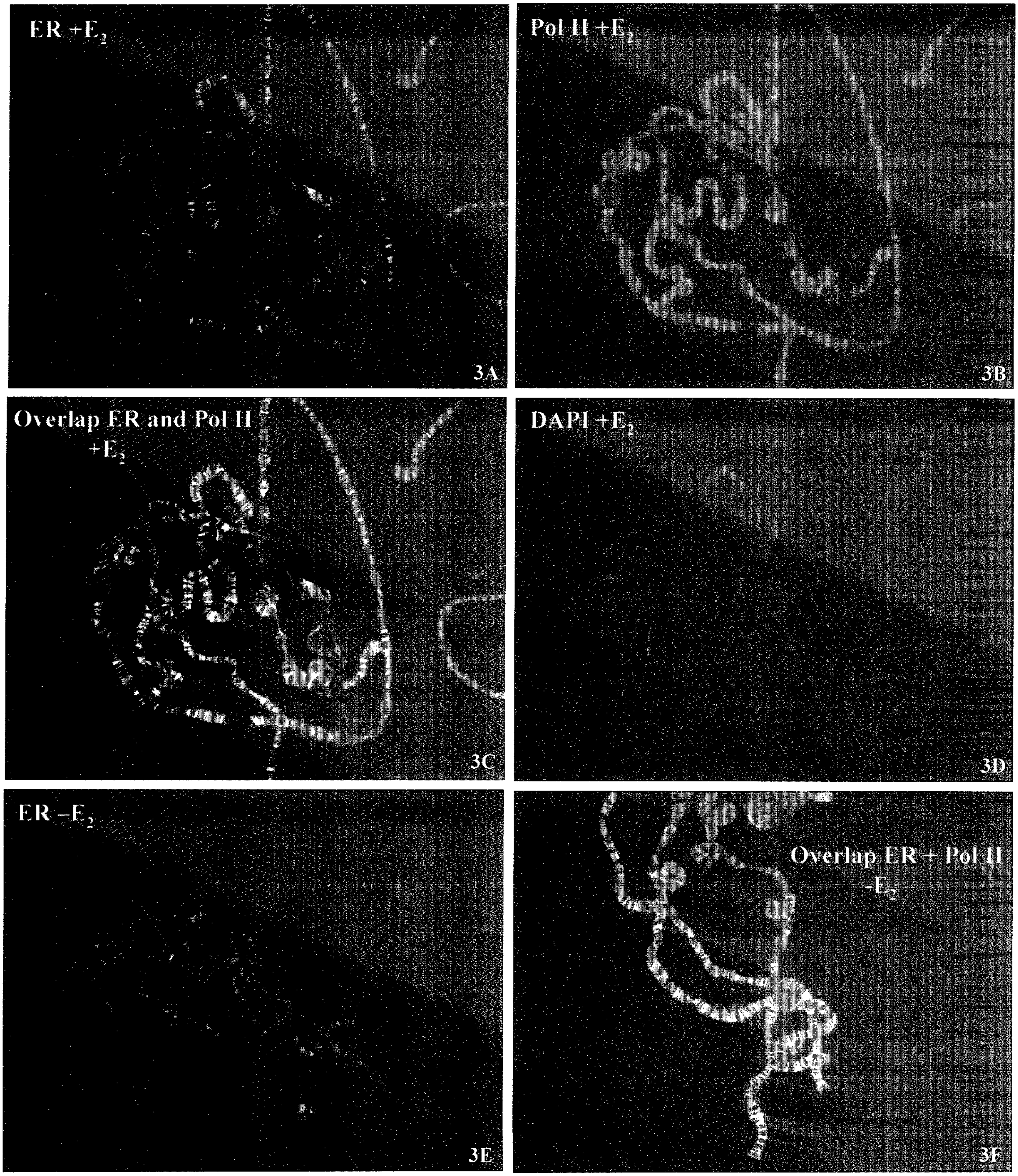
Estrogen dependent GFP expression


ER +E_2
3A
Pol II +E_2
3B
Overlap ER and Pol II +E_2
3C
DAPI +E_2
3D
ER –E_2
3E
Overlap ER + Pol II -E_2
3F